CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 Sampling

A. aegypti is an urban resident known only to survive in fresh water. In order to obtain a more comprehensive and practical investigation, three fresh water sources in the urban settings - rain water, river water and stagnated water bodies - were chosen.

3.1.1 Rainwater

Rainwater was collected from different housing localities. The chosen sampling sites were scattered all over the city (as shown in Figure 3.1)ranging from very well developed housing estates to poorly managed ones as described below. During the period of sampling, there was a thick haze enveloping the Kuala Lumpur city. Rainwater that was collected would contain these pollutants (particulates). The waste disposal facilities and drainage in these places were also important parameters to take into consideration.

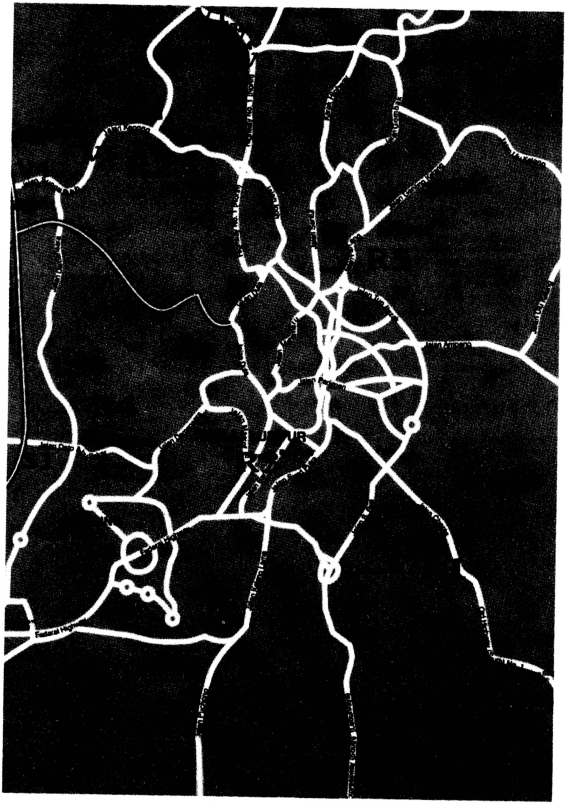

Figure 3.1 : Map Showing the Sampling sites for Rainwater, River Water and Stagnant Water Samples

R1=Cheras Rainwater; R2=Segambut Rainwater;R3=Bangsar Rainwater; R4=Damansara Rainwater; R5=IMR Rainwater; V1=Sungai Kanching (Point A); V2=Sungai Kanching (Point B); V3=Sungai Kanching (Point C); V4=Sungai Keroh(Point A); V5=Sungai Keroh (Point B); S1=Point I; S2=Point II; S3=Point III; S4=Point IV; S5=Point V; S6=Point VI

Rainwater was collected at an open space during a heavy downpour using a clean plastic bucket that was first rinsed in the rainwater. The sample was then transferred via a funnel into 1.5L plastic bottles and labeled with the date and venue and stored at 4°C for further analysis.

3.1.1.1 Cheras Housing Estate

Cheras at 6.5 miles is a fairly well-developed housing estate which houses about 10,000 residents. Most of the housing structures are limited to either semi-detached or link houses allowing close proximity and increasing the chances of contracting dengue. Drainage systems were not clogged up except in certain areas where rubbish had accumulated. This housing estate could be classified as a fairly well-managed one.

3.1.1.2 Segambut Housing Estate

Taman Cuepacs, Segambut, is a housing area that comprises mainly of terrace houses in very close proximity and undeveloped drainage systems. This place was located away from traffic pollution but was exposed to open burning of rubbish in the vicinity which worsened the effect of the haze. The roads and drainage systems were left undeveloped since its construction in the early 80's and waste was disposed at open pits which encouraged the breeding of pests and created a very unhygienic atmosphere.

3.1.1.3 Bukit Damansara Housing Area

This is a well-developed housing area that comprised mainly of bungalow houses at a considerable distance from each other. There was an overgrowth of vegetation in the locality that could pose a threat as a resting place for the adult mosquito. Drainage systems were well maintained and rubbish disposal was well organized. This place was also free from traffic pollution but did not escape the haze in the city.

3.1.1.4 Bangsar Baru Housing Estate

This is a well-structured and well developed housing estate that has close to a few hundred residents. The houses are mainly semi-detached and link houses that have close proximity but considerable privacy. There was much traffic pollution but this was mainly at the main road, a few hundred meters away from the housing estate. The drainage and rubbish disposal systems were well maintained.

3.1.1.5 The Institute of Medical Research, Jalan Pahang.

This locality was in the heart of much traffic pollution mainly buses, cars and lorries that frequently pass this area. Therefore, the air pollution level here was quite unhealthy . Drainage systems were not developed and this caused flooding to occur during a heavy downpour, an ideal condition for larval breeding. Rubbish disposal was also not well-organized, causing bad odour and unhygienic conditions for residents in the vicinity.

3.1.2 River Water

Numerous river channels that flow through the city as shown in Figure 3.1. It is highly unlikely that swift flowing rivers would house *A. aegypti.* However these rivers were heavily clogged with rubbish and polluted with effluents from industries as well as runoff from livestock farms. These created temporary stagnated pools of water that could attract gravid female mosquitoes.The Department of Environment (DOE) is now taking measures to rehabilitate these rivers so that it could be used as water catchment areas. As this study was carried out in late 1997 to early 1998 when Malaysia was facing a drought season due to the after effects of the El Nino phenomenon, the river water was shallow, due to its low water content and there was no free flow. It was this standing water that was sampled for this study.

Two rivers - Sungai Kanching and Sungai Keroh - were sampled. Water was collected from 3 points along Sungai Kanching, Point A (undisturbed rainforest), Point B (Orang Asli settlement) and Point C (livestock farm). At Sungai Keroh, water was collected from 2 points, Point D (FRIM forest reserve) and Point E (polluted river). Water samples were collected midstream where there was uniformity in composition. Sampling was carried out by immersing a plastic bucket that was first rinsed in the river water in the midst of the stream until it was filled. This sample was then transferred via a plastic funnel into 1.5L plastic bottles and labelled with the date, venue and sample collected. The samples were stored at $4^{o}C$ until further analysis. Figure 3.1 shows the river water sampling sites in Sungai Kanching and Sungai Keroh.

3.1.2.1 Sungai Kanching (Point A) - Undisturbed Rainforest

The river, Sungai Kanching, originates from a rainforest commonly known as Templer's Park located at the northern border of Federal Territory and Selangor. There were huge rocks along the river that hindered water flow and causing water stagnation. It was clear and free from pollutants. The shade provided by the vegetation at the banks of this river is definitely suitable for mosquito breeding and as a resting place for the adult mosquito.

3.1.2.2 Sungai Kanching (Point B) - Orang Asli (Aborigine) Settlement

Sampling was carried out at the second point along Sungai Kanching. There is an Orang Asli (aborigine) Settlement at the bank of this river. The residents use the river for washing, bathing and drinking purposes. It is also used for recreation by foreigners and visitors. There was a certain degree of pollution in the river due to rubbish disposal, organic substances and debris but on the outlook it, seemed to be clear.

3.1.2.3 Sungai Kanching (Point C) - Along a Golf Course and Livestock Farm

This point of sampling was downstream of the river Sungai Kanching. At this point the river flowed through a golf course and livestock farm. There was runoff from the farm that caused the river to look murky and the area was cleared to make way for a golf course along its banks. The cattle uses the river for drinking and bathing purposes. The lack of vegetation along the banks of the river causes soil erosion into the river and therefore clogs up the flow in certain points. The river is inaccessible by foot as it is quite deep and dangerous to delve into.

3.1.2.4 Sungai Keroh (Point D) - FRIM Forest Reserve

Sungai Keroh originates from a forest reserve right in the midst of the Klang Valley. This forest reserve belongs to the Forestry Reserve Insitute of Malaysia (FRIM). The river in this place flows through a tropical forest that is used for research and recreation. Tourists frequently visit this place and the residents in the locality use the river for bathing and washing purposes. There is much debris and organic substances in this river. Huge granite rocks along the river causes the river water to stagnate at certain points. River flow was not very swift. There was much vegetation along the banks of the river that hindered direct sunlight and made good shades.

3.1.2.5 Sungai Keroh (Point E) - Polluted River Water

This sampling point was located downstream of the river Sungai Keroh. It flows through the Segambut and Kepong Industrial Area where untreated effluents from the nearby factories are discharged into the stream. Residents at the bank of the river also dump solid waste and organic substances into the river causing the river to give out a foul odor and lowering the river water quality. There is abundant flora and fauna in this river and there is ample shade provided by the vegetation at the banks.

3.1.3 Stagnated Water Bodies

Stagnated water bodies found abundantly in the environment are sites where *A.aegypti* breed. *A. aegypti* has been found to breed abundantly in any form of stagnated water in the environment including tin cans, automobile tyres and jars. Six points were chosen for sampling - Point I (ditches in an abandoned housing project), Point II (swamp adjacent to a school), Point III (pools at a construction site), Point IV(drain in an industrial area), Point V(water collected from a rusty tin can) and Point VI (pond water from a fishing pond).

Figure 3.1 shows the stagnant water sampling sites collected in the various places in the Klang Valley.

3.1.3.1 Ditches In An Abandoned Land - Point I

Due to the 1997 economic downturn, many developers had abandoned their projects and these bare lands were a common sight in the city. Sampling was carried out in one such abandoned area situated in Sri Hartamas. The land which previously was a rubber plantation, was cleared to make way for a housing estate. However, the developer had no resources to continue with the project. Therefore, this land area was not occupied. A heavy shower had formed ditches in the laterite soil and water had stagnated there for a long time. There was an abundance of overgrowth of secondary rainforest that provided ample shade and it seemed to be a perfect spot for mosquito breeding.

Water was sampled in the ditches found in this abandoned land at random using a scoop to transfer the water via a funnel into 1.5L plastic bottles that were first rinsed in the water sample. The bottles were labeled with the date, venue and point of collection. It was stored at 4°C until further analysis.

3.1.3.2 A Swamp adjacent to a school - Point II

As schools housed various residents, it is imperative that its surroundings be *Aedes* free. The site chosen for sampling was beside an International school that is located in a quiet and serene vicinity. There was a swamp about 100m from the school canteen. As swamp water was freshwater and stagnated, it could pose a threat. There was hardly any visible fauna present in this water source that would serve as a predator for the larvae or the eggs of the mosquito therefore it would be an ideal egg-laying site. There were marshes along the bank of the swamp that provided shade and could be a potential resting place for the adult *A. aegypti.*

As the water source was quite deep and inaccessible, a 6L plastic bucket that was fastened securely to a rope was lowered into the swamp where the water composition was uniform. After a few minutes the bucket was the pulled back to the surface and the water sample that had been collected was transferred into1.5L plastic bottles using a funnel. These bottles were labeled with the date, venue and point of collection before being stored at 4°C until further investigation.

3.1.3.3 Pools at a Construction Site - (Point III)

Construction sites are a common sight in the city of Kuala Lumpur. Much land was being cleared to make way for development However, these construction sites were poorly managed and posed a threat to workers and residents in its locality.

Routine government checks have discovered *A. aegypti* larvae in these construction sites in the pools of water created by heavy showers in these areas. Therefore, the investigation of water quality analysis and mosquito breeding and survival in these sites is necessary.

The construction site chosen for this study was surrounded by housing estates that were newly developed. The ground had been broken for setting the foundation and steel skeletons for the upcoming building. During a downpour, the soil was easily excavated as there was a lack of plant roots to hold it together. This caused little pools of water to form at the construction site. Water sampling was carried out in these pools by using a plastic scoop to collect the water and transfer it into 1.5L plastic bottles that were already rinsed in the sample to be collected. There was no visible flora or fauna in the water samples but ample shade was provided by the nearby buildings that could be a potential resting place for the mosquito. The water samples were then stored at 4^oC until further use.

3.1.3.4 Drain in an Industrial Area - Point IV

As heavily clogged drains could provide ideal larval habitats, water sampling was also carried out in a drain adjacent to a cement factory. The drain did not have a free flow and water had stagnated there for a few days. This locality was an industrial area that had many factories in its vicinity and many of its workers could contract dengue. Water was collected from the drain at the mid-point where much water had accumulated and stagnated using a 6L plastic bucket fastened securely to a rope. This bucket was immersed into the water and then pulled up to the surface. The water sample was then transferred into 1.5L plastic bottles that were first rinsed with the sample and labeled with the date, venue and point of collection. These were later stored at 4°C until further use.

3.1.3.5 Water Collected from a Rusty Tin - Point V

As it was a well-known fact that *A. aegypti* is a container breeder, sampling was carried out in a rusty tin can that had collected water after a downpour. This was among the tin cans found lying in the environment that was not properly disposed. Water was transferred from this tin can into 1.5L clean plastic bottles that were first rinsed in the sample. These bottles were labelled with the date, venue and point of sampling. It was then stored at 4°C until further use.

3.1.3.6 Pond Water - Point VI

Lakes and ponds are freshwater sources likely to be inhabited by mosquitoes due to its permanent stagnant state. The organic content of the pond is dependent on the activities of the inhabitants of this water source and is rich with nutrients for the immature *A. aegypti*. The vegetation at the banks usually provide ideal resting places for the adult mosquito and therefore is ideal as an ovipository site.

Water was collected from a pond in a tropical rainforest environment that was rich with organic content. A 6L bucket that was fastened securely to a rope was cast into the midst of the pond where the water content was well-mixed. It was then pulled back to the surface and transferred into two 1.5L plastic bottles using a funnel. These bottles were labeled with the date, venue and sample collected. They were later stored at 4°C until further use.

3.2 PHYSIO-CHEMICAL ANALYSIS OF WATER

Analysis was done in the laboratory to determine certain water quality parameters. The parameters measured are suspended solids, rate of filtration, pH and heavy metal concentrations of arsenic (As), cobalt (Co), chromium (Cr), iron (Fe), manganese (Mn), zinc (Zn), magnesium (Mg), cadmium (Cd), copper (Cu), lead (Pb) and tin (Sn). This physio-chemical analysis was carried out in order to investigate any correlation that existed between these parameters that either supported and enhanced mosquito breeding or inhibited its survival.

3.2.1 Suspended Solids (SS)

Suspended solids in the water sample was measured by running water samples through a funnel that was layered with Whatman filter paper. The weight of the Whatman filter paper was first taken. The solids that were trapped at the end of the filtration was then weighed together with the filter paper. Total amount of dissolved solids in 100ml of sample water was determined by subtracting the weight of the filter paper from the total amount of trapped solids on the filter paper. The unit of measurement for this parameter was g/ml.

3.2.2 Rate of Filtration (ROF)

The rate of filtration was a measurement to determine the time taken for 100mL of a water sample to settle at the end of a filtration. Using a stop watch, time was recorded from the moment the sample was run through a filter paper until the water stopped flowing. The unit of measurement was seconds.

3.2.3 pH

To investigate the acidity or alkalinity of a water sample, pH of the sample was taken using a pH meter. The electrode inside the glass rod probe was first calibrated using 2 solutions, one of pH 4 and the other of pH 7. pH of the water sample was recorded after the reading stabilized.

3.2.4 Heavy and Trace Metal Concentration

Heavy and trace metal concentrations was determined from the water samples that were taken from the environment. This was carried out using the ICP-AES analyzer which is a very sensitive instrument used to detect trace and heavy metals in water samples. The unit of measurement for the trace and heavy metals was in parts per million (ppm). In this method, the heavy or trace metal elements in a particular water sample would emit rays at a certain wavelength that was detectable by the ICP-AES analyser. From the standard curve of each element (that measures wavelength against concentration), the concentration of each element in the water sample can be determined. Appendix I – VII show the standard curves for certain elements that were measured using this method.

3.3 Monitoring the Breeding and Survival of *A. aegypti*

This part of the study was conducted in the Institute of Medical Research, Jalan Pahang. The purpose of this study was to investigate the effect of different water samples on the growth and survival of *A. aegypti*. The hatching rate that represents the percentage of eggs that actually hatched into first instar larva from the number of eggs originally immersed was determined on the third day from the start of the experiment The larval survival rates for the first, second and third instars that corresponded to the fifth, seventh and tenth day from the start of the experiment were determined. The rates of survival of pupae into adult stages were noted at the pupal and adult stages - on the fourteenth and eighteenth day from the start of the experiment, respectively.

3.3.1 Hatching Rate of *A. aegypti*

The hatching rate of *A. aegypti* refers to the number of eggs that hatched into larva as a percentage of the total number of eggs that were initially immersed in the water sample. One hundred eggs that were preserved by dessication on Whatman filter paper were immersed in the water samples. The water samples were placed in uniform, plastic containers and covered with wired mesh to allow for aeration and to prevent other mosquitoes from egg-laying.. Larval feed in the form of grounded rat's liver was scattered on the surface of the water sample. A total of three replicates were used for each of the 16 water samples and tap water was used as a control. At the end of 3 days, all eggs that hatched into first instar larva from the total amount of eggs immersed in the water was calculated and recorded.

3.3.2 Larval Count at Three Stages

Larval count was taken for each triplicate of the water sample and the control water at the second and third instar stages of the larvae. The count was taken on the fifth and seventh day from the start of the experiment. The number of larvae that survived to the second instar stage (on the fifth day of the experiment) were counted and recorded as a percentage of first instar larva that survived on the third day of the experiment. The number of third instar larvae that survived on the seventh day from the start of the experiment was taken and recorded as a percentage of the total number of second instar larvae that survived.

3.3.3 Pupae Count and Survival

At the tenth and fourteenth day from the start of the experiment, the total number of pupae that survived were counted. The count was first taken on the tenth day from the start of the experiment to mark the initial number of pupae from the final larval instar stage. During this time, there were still third stage larval instars that had not entered the pupal stage. On the fourteenth day from the start of the experiment, the total number of pupae was taken. The pupae was then transferred into clean, plastic bowls and placed in wooden cages to prepare for the adult stage. Pupa count was first taken and recorded on the tenth day from the start of the experiment as a percentage over the total number of third instar larvae that survived (on the seventh day). The number of total surviving pupae was taken on the fourteenth day as a percentage of the number of survivors on the tenth day of the experiment.

3.3.4 Adult Count

On the eighteenth day from the start of the experiment, when the pupae that survived had emerged into adult *A. aegypti*, a muslin cloth dipped in sugar solution and fastened on a stand was placed in the cage as feed for the adult mosquitoes. The number of surviving adult mosquitoes at the end of 18 days were counted as a percentage of the total number of pupae that survived on the fourteenth day from the start of the experiment.

3.4 Statistical Analysis

3.4.1 Principal Component Analysis

The relationships between the physio-chemical parameters (pH, suspended solids, rate of filtration, heavy and trace metals concentrations) and the respective location of sampling sites were studied using an ordination technique, principal component analysis (PCA) . This was carried out using the computer software Statistica Version 5.0 (Factor Analysis). Basically, the technique reduces the number of variables in the data set by finding linear combinations of those variables that best explain most of the observed variability.

The extraction of principal components amounts to a variance maximizing (Varimax) rotation of the original variable space. This type of rotation is called variance maximizing because the criterion for the rotation is to maximize the variance of the extracted factor while minimizing the remaining variability. The number of factors to extract depends on the point when there is only very little random variability left as the more consecutive factors are extracted, the less variability is accounted for.

Scatterplots (or biplots) of cases (in this study - the source of water samples), were then drawn using the pairs of principal components that were extracted as axes. Since the factors are not correlated, such scatterplots will not give any information about linear dependencies but nonlinear structures in the data set can be revealed to show the similarities in composition of water samples collected from different localities.

3.4.2 Instantaneous Mortality Rate

To investigate further on the survival of *A. aegypti* in different water samples, a decaying exponential model was used to fit the observed population data. The mortality, which is the inverse of survival was calculated from the following equation :

$$N_t = Noe^{-zt}$$

where N_o = initial population size, N_t = population size at time t (numbers surviving to time t) and z= instantaneous mortality rate (Pauly, 1978). Therefore, z can be estimated using linear regression analysis from the logarithmic transformation

$$\ln N_t = \ln N_o + (-z)t$$

where z is the slope obtained by regressing ln Nt on t. The instantaneous mortality rate (z) of immature and adult forms of *A. aegypti* was determined for each water sample.

The null hypothesis $H_o : z_1=z_2=z_3..........z_k$ (no difference in mortality among the k water samples) was tested against the alternate hypothesis H_A : at least one pair of z's are not equal. To test the null hypothesis against the alternate hypothesis, an analysis of variance(ANOVA) was used. This hypothesis was tested at the 5% level of significance. If the null hypothesis was rejected, significant differences among the mortality rates of larvae bred in the 16 different water samples were tested using an analysis of covariance, a procedure used for multiple comparisons of more than 2 slopes (Zar, 1984). As a result of this analysis, statistical significance could be determined between mortality rates in any two of the water samples.

3.4.3 Percentage Mortality

The percentage of mortality of *A. aegypti* at three phases of development, egg (embryonic), larval and pupal stages was determined. However, as there were 3 replicates involved for each of the 16 water samples, the mortality rate at each phase was the average rate for the 3 replicates. At the embryonic phase, the total number of eggs that failed to hatch from the total number of eggs used will be the percentage of mortality. That is,

$$\frac{\text{Total no. of eggs - total no. of eggs hatched}}{100} \times 100\%$$

For the larval phase, the total number of larva that failed to pupate was taken as a percentage from the total number of first instar larva that had emerged from the egg. That is,

$$\frac{\text{Total no. of } 1^{st} \text{ instar larva} - \text{total no. of } 4^{th} \text{ instar larva}}{\text{Total no. of } 1^{st} \text{ instar larva}} \times 100\%$$

For the pupal phase, the number of pupa that failed to emerge into adult mosquitoes were expressed as a percentage of pupa that survived from the larval phase. That is,

$$\frac{\text{No. of pupa on the } 14^{th} \text{ day - no. of adults on } 18^{th} \text{ day}}{\text{Total no. of pupa on the } 14^{th} \text{ day}} \times 100\%$$

3.4.4 Correlation Analysis

Pearson's correlation analysis was carried out to determine if any correlation existed between the mortality data after arcsine transformation (Zar, 1984)and the water quality parameter results obtained during the laboratory analysis. Arcsine transformation of data was necessary because the mortality data was obtained in percentage. A correlation matrix of all variables was first constructed and examined for significant relations using the computer software Statistica (Version 5.0).

3.4.5 Multiple Regression Analysis

To study the functional relationships between mortality and water parameters, a multiple regression analysis was carried out. The computer software Statistica Version 5.0 (multiple regression analyses) and the forward stepwise routine was used for this analysis. The general purpose of multiple regression is to analyze the relationship between several independent variables and a dependent variable. In this case, the dependent variable is the percentage of mortality at each phase of development and the independent variables are the water quality parameters namely pH, suspended solids, rate of filtration and the concentration of the 11 different elements in the 16 water samples. In a multivariate case like this a linear equation has to be constructed that contain all these variables. Generally, multiple regression procedures estimate a linear equation of the form :

$$Y = a + b_1x + b_2x + \dots\dots\dots\dots b_px$$

The regression coefficients (or b coefficients) represent the independent contribution of each independent variable to the prediction of the dependent variable. The regression line expresses the best prediction of the dependent variable (Y) given the independent variables (X). The deviation of a particular point from the regression line is the residual value. The smaller the variability of the residual values around the regression line to the overall variability, the better the prediction. The R squared value (coefficient of determination) is an indication of how well the model fits the data (an R squared value close to 1.0 indicates that the model accounts for almost all of the variability in the respective variables). The degree to which 2 or more predictions are related to the dependent variable is expressed in the multiple correlation coefficient R.

Multiple regression analysis was done separately for each phase of development (embryonic, larva and pupa) based on the mortality data (dependent variable) after arcsine transformation and water quality parameters (independent variables) obtained for the 16 water samples.